Human Breast Cancer Cell Cycle Synchronization by Estrogens and Antiestrogens in Culture

C. Kent Osborne, David H. Boldt, and Phillip Estrada
Department of Medicine, University of Texas Health Science Center, San Antonio, Texas 78284

ABSTRACT

The mechanisms by which estrogens and antiestrogens modulate breast cancer growth have not been totally defined. We have examined the cell cycle kinetic effects of estrogens and antiestrogens in cultured human breast cancer cell lines. In a previous study, we showed that tamoxifen induces a transition delay in early to mid-G1 phase of the cell cycle. In the present study, we show that this cell kinetic alteration by tamoxifen is dose dependent and that other antiestrogens have identical effects. As little as 0.01 to 0.1 μM tamoxifen reduces the S and G2-M fractions and increases the G1 fraction of MCF-7 cells growing in medium with 5% charcoal-stripped bovine serum. More than 90% of cells are in G1 72 to 96 hr after the addition of 1 μM tamoxifen, a concentration achieved in patients treated with the drug. Nafinoxidine and trioxifene have identical activity. Partial reversal of tamoxifen growth inhibition is observed with a simple change to tamoxifen-free medium. Complete reversal of the tamoxifen effect is observed with the addition of 17β-estradiol. By 24 hr after the addition of estrogen, 60 to 70% of tamoxifen-inhibited cells have progressed through G1 and into S phase, indicating that tamoxifen-treated cells remain viable. This estrogen "rescue" effect is observed even in a change to tamoxifen-free medium. A 100-fold-lower concentration of estradiol can totally reverse the inhibitory effects of 1.0 μM tamoxifen. Stimulation of the progression of G1 cells to enter S phase is also observed when estradiol is added to cells maintained for four days in medium with stripped serum, even in the absence of tamoxifen. Similar effects are observed in the estrogen receptor-positive ZR75-1 breast cancer cells. No effects of antiestrogens or estrogens are observed in the receptor-negative MDA-231 cells, suggesting that these effects are mediated through the estrogen receptor. In summary, antiestrogens and estrogens have prominent effects on the cell cycle kinetics of endocrine-dependent human breast cancer cells. Antiestrogens cause an accumulation of cells in G1 phase. Estrogen reverses this block with a synchronous cohort of cells progressing through the cell cycle. These data have important implications for the design of rational clinical trials of combined chemoendocrine therapy.

INTRODUCTION

Antiestrogens are widely used for palliation of women with hormone-responsive human breast cancer. More recently, antiestrogen treatment has been combined with cytotoxic chemotherapy in both patients with metastatic disease and those undergoing adjuvant therapy following mastectomy with the hope of achieving additive antitumor activity. Although preliminary results suggest that certain subgroups of patients might benefit from the combined approach (12, 27), other studies report no significant response or survival improvement (4-7), or even a deleterious effect of combining the antiestrogen tamoxifen with cytotoxic agents (12). The lack of the expected additive effects suggests that tamoxifen may have an antagonistic interaction with the cytotoxic agents or with the host (5, 20).

The mechanisms of antiestrogen action are not well defined. Antiestrogens can bind to and translocate cytoplasmic estrogen receptor to the nucleus, where presumably they inhibit estrogen-mediated events leading to tumor growth (13). Several estrogen-sensitive processes are inhibited by exposure of breast cancer cells to antiestrogens (10, 14-16), but the net effect of these biochemical events on cell proliferation and specifically on cell cycle kinetics is not clear.

Recent reports suggest, however, that antiestrogens have profound effects on breast cancer cell cycle kinetics that could partially explain the possible antagonism observed in the clinical trials of chemoendocrine therapy. Incubation of human breast cancer cells with concentrations of tamoxifen achieved in vivo results in accumulation of cells in early G1 phase (21, 25, 26), where they may be less susceptible to the lethal effects of cycle-active cytotoxic drugs. We now extend these initial observations and show that (a) other antiestrogens, in addition to tamoxifen, block cell cycle progression in G1; (b) the cell cycle block can be reversed by incubation with estrogen, resulting in a synchronized cohort of cells progressing through S phase; and (c) the cell cycle effects are not observed in estrogen receptor-negative cells, suggesting that the effects are mediated via the estrogen receptor.

MATERIALS AND METHODS

Breast Cancer Cells and Culture Techniques. The human and mammary origins of the MCF-7, ZR75-1, and MDA-231 breast cancer cell lines have been described previously in detail (18). The MCF-7 and ZR75-1 cells contain estrogen receptor, whereas the MDA-231 cells do not. The cell culture techniques used for the maintenance of these cells have been reported previously (23).

Hormones. Tamoxifen citrate was generously provided by Stuart Pharmaceuticals, and nafioxidine, by The Upjohn Co. Trioxifene (LY 133314) was the generous gift of Lilly Research Laboratories. 17β-Estradiol was purchased from Calbiochem (San Diego, CA).

Cell Kinetic Studies. Breast cancer cells were plated into 100-mm culture dishes (Corning Glass Works, Corning, NY) in complete culture medium (Richter's Improved Eagle's Minimal Essential Medium-ZG; Grand Island Biological Co., Grand Island, NY) supplemented with insulin (1.0 μg) and 5% bovine serum (MCF-7 and ZR75-1 cells) or 10% fetal bovine serum (MDA-231). After the cells had firmly attached to the dish (24 hr), complete medium was replaced with identical medium supplemented only with 5% bovine serum stripped with dextran-coated charcoal to reduce the endogenous estrogen concentration. After an additional 24 hr, hormones or ethanol (vehicle control, final concentration =
0.1%) was added directly to the culture dishes.

The TLI was performed by a modification of the method of Livingston et al. (19). Cells were incubated for a 30 min pulse with tritiated thymidine (47 Ci/mmol; Amersham) at a final concentration of 0.3 μCi/ml, washed 3 times, suspended in PBS with 0.02% EDTA, and centrifuged (600 rpm for 2 min). Cells were resuspended in PBS (2.5 × 10^6 cells/ml), and 0.2 ml of the suspension was cytocentrifuged for 5 min at 1000 rpm. After drying, slides were fixed, developed, and stained as described previously (19). A total of 500 cells was counted for each point. The TLI (labeled/total cells) was calculated by counting the fraction of intact cells containing 8 or more grains over the nucleus.

Cell cycle distributions were obtained by computer analysis of DNA histograms obtained by flow cytometry of mithramycin-stained cells (1, 9, 21). Cell monolayers were suspended in 0.02% EDTA in PBS and incubated for 10 min, and then a single-cell suspension was prepared by passing the cells through 22-, 25-, and 27-gauge needles. Cells were pelleted, resuspended in PBS, and fixed in 70% ethanol (final concentration) while vortexing. The fixed cells were pelleted, washed once in PBS, and stained overnight in the dark at 4°C with mithramycin as described previously (1, 21). Prior to flow cytometry, the cell suspension was again passed through a 27-gauge needle and through a 60-μm nylon mesh screen to remove clumps. Doublets constituted less than 3% of the total cell suspension as determined by light microscopy. Histograms were obtained by analyzing 1 × 10^6 cells on a Coulter Model TPS-1 cell sorter equipped with a 2-watt argon ion laser tuned to 457 nm. The coefficient of variation of the d peak varied from 4 to 8% using this method.

RESULTS

Cell Cycle Effects of Antiestrogens. Initially, the TLI was used to estimate the fraction of cells actively synthesizing DNA after incubation with tamoxifen. After 96 hr with 1 μM tamoxifen, a concentration readily achieved in patients administered standard doses (11), the TLI was reduced in MCF-7 cells growing in serum-free medium or in medium supplemented with 5% stripped serum (Table 1). Although the effects of tamoxifen were slightly more pronounced under serum-free conditions, all subsequent experiments were conducted with cells maintained in medium containing stripped serum, conditions in which near-optimal cell proliferation is maintained.

It has been reported previously that the reduction in the S-phase fraction of MCF-7 cells with tamoxifen is accompanied by an increase in the G1 fraction (21, 25, 26). Flow cytometry of cells stained with the DNA fluorochrome mithramycin revealed that this antiestrogen effect is dose dependent (Chart 1, A and B). Concentrations of tamoxifen between 0.01 and 1.0 μM resulted in a stepwise reduction in the fraction of cells in S and G2+M phases and a concomitant accumulation of cells in G1. Fewer than 10% of cells were in S phase, whereas greater than 90% were in G1 after exposure to 1.0 μM tamoxifen.

This antiestrogenic effect was also time dependent and was observed with other antiestrogens as well as tamoxifen (Chart 2). After a 96-hr exposure of MCF-7 cells to tamoxifen, nafloxi-dine, or trioxifene, more than 90% of cells were in G1 phase compared to 65% in control dishes. There was a concomitant reciprocal reduction in the fraction of cells in the S and G2+M phases observed with the antiestrogens.

Effect of Estrogen. An important question is whether or not the cells accumulating in G1 phase after antiestrogen exposure are destined to die. Previous reports have demonstrated that cell proliferation can be restored by the addition of 17β-estradiol to tamoxifen-treated cultures (8, 15, 16). We observed a dose-dependent ability of 17β-estradiol to recruit tamoxifen-treated MCF-7 cells into S phase (Chart 3). After 72 hr in tamoxifen, the TLI (S fraction) was less than 10%. At that time, medium was changed to fresh medium (with 5% stripped bovine serum) supplemented with increasing concentrations of estradiol. A simple change to tamoxifen-free medium resulted in a significant increase in the TLI in 24 hr. This stimulation of cells into S phase was probably related to replenishment of essential nutrients or other growth factors, rather than removal of tamoxifen, since a change to fresh medium containing tamoxifen resulted in a similar pattern (see below). With the addition of estradiol to the tamoxifen-inhibited cells, a further increase in the fraction of S-phase cells at 24 hr was evident. Even 0.1 μM estradiol caused a modest increase in the TLI above that observed with a change in medium alone. Maximal effects were observed with 10 nM estradiol; 65% of cells were in S phase.

To study the effects of estrogen replenishment in more detail, we studied the cell cycle phase distributions of tamoxifen-inhibited cells supplemented with estradiol and analyzed by flow cytometry (Charts 4 and 5). DNA histograms (Chart 4) demonstrate that a synchronous wave of cells entered and passed through the S phase between 12 and 24 hr after adding estradiol. Chart 5 displays in more detail the time course of the effects of estrogen on control and tamoxifen-inhibited cells analyzed by flow cytometry. A sharp drop in the S fraction with a concomitant increase in the G1 fraction was observed with a 96-hr exposure to tamoxifen. The S fraction also declined somewhat in control cultures, probably secondary to nutrient depletion. At 96 hr, medium was changed to fresh medium alone (control) or medium containing estradiol or tamoxifen. In both control and tamoxifen-inhibited cultures, a simple change in medium, even medium containing tamoxifen, resulted in a significant fraction of cells leaving G1 phase and passing through the S compartment. The addition of estradiol to tamoxifen-treated or control cells caused a greater increase in the S fraction which peaked at about 70%. The S fraction in control cells treated with a medium change was about 30% higher than in cells treated with estradiol only. The lag time was longer in tamoxifen-inhibited cells rescued by estradiol; the maximal increase in S fraction in these cells required 18 hr. This difference in the timing of the estrogen "rescue" effect on control or tamoxifen-pretreated cells was consistent in several experiments.

Control or tamoxifen-treated MCF-7 cells can be stimulated to enter S phase by estrogen without a change to fresh culture medium (Chart 6). In this experiment, cells were incubated in the absence of hormone (controls) or with tamoxifen for 72 hr.  

<table>
<thead>
<tr>
<th>Condition</th>
<th>TLI at 96 hr (%)</th>
</tr>
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<tbody>
<tr>
<td>Control, stripped serum</td>
<td>25</td>
</tr>
<tr>
<td>Control, serum free</td>
<td>17</td>
</tr>
<tr>
<td>Tamoxifen, stripped serum</td>
<td>5</td>
</tr>
<tr>
<td>Tamoxifen, serum free</td>
<td>3</td>
</tr>
</tbody>
</table>

The abbreviations used are: TLI, tritiated thymidine labeling index; PBS, Dulbecco’s phosphate-buffered saline without calcium or magnesium.
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Chart 1. Effect of tamoxifen (TAM) concentration on DNA histograms obtained by flow cytometry of mitramycin-stained MCF-7 cells. Cells were grown in 5% charcoal-stripped bovine serum with increasing concentration of tamoxifen for 96 hr. A single-cell suspension was prepared, fixed, stained, and analyzed as described in “Materials and Methods.” A. histograms obtained from analysis of 1 x 10⁶ cells. The coefficient of variation of the G₁ peaks varied from 4 to 8%. B, G₁ and S fractions computed from the histograms and plotted in chart form.

Chart 2. Time course of the effect of antiestrogens on MCF-7 cell cycle kinetics. MCF-7 cells were cultured in control medium (•) or medium containing 1 μM tamoxifen (O), nafoxidine (D), or trioxifene (A). Cell cycle phase distributions were determined as described in “Materials and Methods.”

Chart 3. Effect of estrogen (E₂) on the TLI of tamoxifen-treated cells. MCF-7 cells were incubated with tamoxifen (1 μM, •) for 72 hr. Spent medium was exchanged for fresh medium supplemented with 5% charcoal-stripped serum and increasing concentrations of 17β-estradiol (O). After 24 hr, the TLI (S phase) was determined.

Estradiol was then added directly to the cultures. Cells left G₁ phase and traversed S phase in a sequence similar to that noted above, with tamoxifen-pretreated cells demonstrating a longer lag time. Since cells could be stimulated by estradiol to enter S phase after growing for several days in medium with charcoal-stripped bovine serum, we also examined the effect of adding 17β-estradiol to cells at the beginning of the experiment (time = 0 hr). Interestingly, initial exposure of MCF-7 cells to 10 nm estradiol resulted in a modest decline in the S fraction and an...
increase in the $G_1$ fraction by 72 hr. Additional estrogen at that point had no effect on the cell cycle distribution, as the majority of cells remained in $G_1$ phase.

**Effect of Estrogen and Antiestrogen on Other Breast Cancer Cell Lines.** We next examined the effects of tamoxifen and 17$\beta$-estradiol on the cell cycle kinetics of 2 other human breast cancer cell lines, the ZR75-1 and the MDA-231. The ZR75-1 cells contain estrogen and progesterone receptors; the MDA-231 cells contain glucocorticoid receptors but lack receptors for estrogen and progesterone (18). The cell kinetic effects of estrogen on the ZR75-1 cells were similar to those described above for the MCF-7 cells (Chart 7). These cells proliferate more slowly than do MCF-7 cells, and only about 10% of cells were in $S$ phase at time 0. A 96-hr exposure to tamoxifen did not significantly alter the cell cycle distribution of these cells. However, as with the MCF-7 cells, estrogen added to controls or to tamoxifen-treated ZR75-1 cells resulted in an increase in the $S$ compartment and a decrease in the $G_1$ compartment at 24 hr. A medium change alone had no effect on the cell cycle phase distribution.

No significant cell kinetic effects of either tamoxifen, estrogen, or a change to fresh medium were observed in the MDA-231 cells (Chart 8). These cells proliferate more rapidly than do MCF-7 cells or ZR75-1 cells, and a relatively low proportion were in $G_1$ at the beginning of the experiment. The fraction in $G_1$ gradually increased with time as the cells neared confluence.

**DISCUSSION**

Previous studies have shown that tamoxifen slows macromolecular synthesis and proliferation of MCF-7 human breast cancer cells growing in medium with charcoal-stripped serum (8, 15, 16). Growth could be restored by the addition of 17$\beta$-estradiol. The timing of this estrogen "rescue" effect was interpreted to indicate that tamoxifen might arrest cells in a uniform position within the cell cycle. The present data extend the previous work reported by Sutherland and coworkers (25, 26) and by our own laboratory (21), demonstrating that tamoxifen-treated MCF-7 cells accumulate in the $G_1$ phase of the cell cycle. This effect is not restricted to tamoxifen but is also observed with other antiestrogens, including nafoxidine and trioxifene. Furthermore, concentrations of tamoxifen observed in breast cancer patients (11) are effective, supporting the notion that this mechanism of inhibition of cell proliferation may be operative in vivo.

Our data demonstrate that tamoxifen inhibition can be reversed by the addition of 17$\beta$-estradiol at a 100-fold-lower concentration. Estrogen-treated cells progress through $G_1$ and into $S$ phase. Furthermore, transit through the cell cycle can be restored by estrogen even when the antiestrogen remains in the culture medium. This is not surprising, since 17$\beta$-estradiol has more than 100-fold-greater affinity for the estrogen receptor than does tamoxifen (8, 16) and since estradiol can compete with tamoxifen and nafoxidine for the nuclear estrogen receptor with restoration of receptor processing and other estrogen events (14). Effects of estrogen are also observed in the estrogen receptor-positive ZR75-1 cells but not in the receptor-negative MDA-231 cells, further suggesting that these cell cycle effects are mediated through the estrogen receptor.

We consistently observed that the lag time between estrogen addition and the maximal accumulation of cells in $S$ phase is longer for tamoxifen-treated cells than for controls. It is doubtful that this increased lag time is due to the time required for
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Chart 5. Time course of the effect of tamoxifen and estrogen on MCF-7 cell cycle kinetics. Cells were incubated in control medium (•) or with tamoxifen (1 μM, O) for 96 hr. Medium was then changed to fresh control medium (•, O), medium containing 1 μM tamoxifen (•, O), or medium containing 10 nM estradiol (A, A). Cell cycle phase analysis was performed at the times shown. Estrogen to enter cells, replace tamoxifen on the nuclear estrogen receptor, and initiate transcription. These events require only a few min (14). However, this observation is consistent with our previous report demonstrating that MCF-7 cells treated with tamoxifen accumulate in early to mid-G1 phase compared to late G1 for control cells (21). Tamoxifen-inhibited cells released by the addition of estrogen would then require more time to traverse G1 phase before entering the S compartment.

We also observed that the antiestrogen block could be partially overcome by changing to fresh medium without or even with tamoxifen. This indicates that other factors present in medium and/or serum (nutrients, growth factors, etc.) are potent growth regulators that can modulate the inhibitory properties of antiestrogens. It is also possible that conjugated estrogens present in "stripped" serum account for the observed effect. This seems unlikely, however, since the effect of fresh serum is observed even when tamoxifen is added at concentrations that should block the effects of low concentrations of estrogen. A more pronounced rescue effect, however, is noted when estradiol is present in the medium. Clearly, the effect of estrogen does not require fresh medium or serum factors, since the addition of estrogen stimulated progression of tamoxifen-blocked cells into G2M phase even in the absence of a medium change.

Chart 6. Effect of tamoxifen and estrogen without a change in culture medium on MCF-7 cell cycle kinetics. Cells were grown for 72 hr in control medium (•), medium with 10 nM estradiol (A), or medium with 1 μM tamoxifen (•). Then, 10 nM estradiol (A, A) was added directly to all wells without a change to fresh medium. Cell cycle phase analysis was performed at the times shown.

S phase even in the absence of a medium change.

It is interesting that no stimulation of cells to enter S phase is observed when estradiol is added to cells shortly after plating. In fact, there is a slight reduction in the S fraction after several
and can be recruited back into the proliferative pool by the addition of estrogen. (c) Our preliminary studies of MCF-7 cells growing as s.c. tumors in immune-deficient athymic nude mice show that prolonged treatment with estrogen depletion and/or antiestrogen results in a block of tumor growth but not in tumor regression (22). Furthermore, tumor growth resumes when estrogen is replenished, even after several weeks of estrogen deprivation or tamoxifen treatment. Tumor regression in patients treated with tamoxifen may result from a block of cell proliferation combined with a functioning host defense system.

Our results have potentially important implications for the treatment of patients with breast cancer, especially concerning the use of antiestrogen therapy combined with cytotoxic agents. The ability to synchronize the endocrine-sensitive cells in G1 phase with tamoxifen or in S phase with short-term estrogen therapy could be used to design more rational treatment strategies. Cytotoxicity of drugs effective in G1 phase might be enhanced by combined therapy with tamoxifen. Alternatively, the activity of S-phase-specific drugs might be synergistically enhanced by synchronization of tumor cells into S phase with estrogen. Preliminary results using laboratory models and clinical trials of patients with breast cancer support these hypotheses (2, 3, 17, 28).

REFERENCES

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